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A ZOONOTIC GENOTYPE OF *ENTEROCYTOZOON BIENEUSI* IN HORSES

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ABSTRACT: This is the first report of *Enterocytozoon bieneusi* in an equid species. Feces from 195 horses from 4 locations in Colombia were examined for *E. bieneusi* by polymerase chain reaction. Of these, 21 horses (10.8%) were found positive for *E. bieneusi*. The prevalence of *E. bieneusi* in horses <1 yr of age was significantly higher (23.7%) than in horses >1 yr of age (2.5%). No significant differences in prevalence were observed between male (13.7%) and female horses (9%). Sequencing of the internal transcribed spacer region of the SSUrRNA locus identified 3 genotypes. Two genotypes appear to be unique to horses and were named Horse 1 and Horse 2. A third genotype, identified as genotype D, was detected in 4 horses. This genotype, previously reported to infect humans, beaver, cattle, dogs, falcons, foxes, macaques, muskrats, pigs, and raccoons, is the most ubiquitous of the *E. bieneusi* zoonotic genotypes. Our findings indicate that *E. bieneusi* from horses can be a potential source of infection for humans.

Microsporidia are obligate, intracellular parasites consisting of more than 1,200 species in 143 genera. They infect a wide range of invertebrate and vertebrate hosts. Of 14 species in 6 genera reported to infect humans, *Enterocytozoon bieneusi* is recognized as the most common microsporidian species (Didier and Weiss, 2006). *Enterocytozoon bieneusi* has been also detected in a broad range of animals, raising the concern that animals could be a potential source of infection for humans. Considerable genetic diversity within *E. bieneusi* has been observed, with more than 80 genotypes of the parasite differentiated based on polymorphisms of the internal transcribed spacer (ITS) sequence of the rRNA gene; some genotypes appear to be host specific, while others have a broad host range, supporting the likelihood of zoonotic transmission (Santín and Fayer, 2009). The only published reports of testing for microsporidia in equids were of horses and donkeys in Switzerland and Spain, which were found to be negative for the parasite (Breitenmoser et al., 1999; Lores et al., 2002). The present study was conducted to examine horses from a different geographic location, Colombia, South America, for the presence of *E. bieneusi* in their feces.

MATERIALS AND METHODS

Source of specimens

Feces were collected by veterinarians from 195 horses from 4 geographic regions in Colombia, South America (Bogota D.C., Sabana de Bogotá, Llanos Orientales, and Costa Atlántica) over a period of 60 days from August to October 2007. Feces collected directly from the rectum of each horse were placed in a 50-ml centrifuge tube. Tubes were labeled, sealed, cooled, and shipped in an insulated container with cold-packs overnight to the U.S.D.A. Laboratory, Beltsville, Maryland.

Recovery of parasites from feces

Spores of *E. bieneusi* were concentrated from feces as described (Fayer et al., 2000). After 15 g of feces from each horse was thoroughly mixed with 35 ml of dH_2O, the suspension was passed through a 45-μm pore size screen. The filtrate, brought to a final volume of 50 ml with dH_2O, was centrifuged at 1,800 *g* for 15 min. The supernatant from each tube was aspirated, and the pellet was resuspended in 50 ml of a 1:1 mixture of dH_2O:CsCl (1.4 g/L) and centrifuged at 300 *g* for 20 min. Supernatant was aspirated from each tube, saved, and washed twice with dH_2O. The final pellet was subjected to DNA extraction.

DNA extraction

Total DNA was extracted from each CsCl-cleaned fecal specimen using a DNeasyTissue Kit (Qiagen, Valencia, California) with a slightly modified protocol. Reagents were provided by the manufacturer. A total of 50 μl of extracted DNA was suspended and thoroughly mixed in 180 μl of ATL buffer. Twenty microliters of proteinase K (20 mg/ml) was added to this suspension and thoroughly mixed. Following overnight incubation at 55 C, 200 μl of AL buffer was added. Manufacturer's instructions were then followed, with the exception that the nucleic acid was eluted in 100 μl of AE buffer to increase the quantity of recovered DNA.

Gene amplification and sequencing

PCR amplification was performed using a set of nested primers, specific for *E. bieneusi*, that amplify the ITS region along with a portion of the flanking large and small subunit ribosomal RNA genes (~400 bp). The outer primers were EBITS3 (5′-GGTCATAGGGATGAAGAG-3′) and EBITS4 (5′-TTCGAGTTCTTTCGCGCTC-3′), and the inner primers were EBITS1 (5′-GCTCTGAATATCTATGGCT-3′) and EBITS2.4 (5′-ATCGCCGACGGATCCAAGTG-3′) as described by Buckholt et al. (2002). The reaction mixture (50 μl) consisted of 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each forward and reverse primer, 2.5 U of *Taq* (Qbiogene Inc., Carlsbad, California), and 2.5 μl of BSA (0.1 g/10 ml). After denaturation at 94 C for 3 min, the first PCR samples were amplified through 35 cycles (denaturation at 94 C for 30 sec, annealing at 57 C for 30 sec, and elongation at 72 C for 40 sec). There was a final extension at 72 C for 10 min. Conditions for the secondary PCR were nearly the same as for the primary PCR; however, there were only 30 cycles and the annealing temperature was 55 C. Fragments of 435 and 390 bp, respectively, were produced.

All PCR amplicons were purified using EXO-SAP enzyme (USB Corporation, Cleveland, Ohio). Purified PCR products were sequenced on both strands with the same primers used for the secondary PCR amplification using Big Dye™ chemistries and an ABI3100 sequence analyzer (Applied Biosystems, Foster City, California). Sequence chromatograms from each strand were aligned and inspected using Lasergene software (DNASTAR, Inc., Madison, Wisconsin). Sequences of these fragments were compared with sequences in the GenBank database by BLAST analysis. GenBank accession numbers assigned to the nucleotide sequences determined in this study are as follows: genotype Horse 1, GQ406053; genotype Horse 2, GQ406054; and genotype D, GQ406055.

Phylogenetic analysis

The ITS rRNA sequences obtained in this study were compared with sequences from other *E. bieneusi* genotypes from GenBank. MUSCLE was employed to construct a multiple sequence alignment of the resulting 92 taxa (Edgar, 2004). The results of computationally efficient means of reconstructing phylogenetic interrelationships were compared to more-formally justified, but time consuming, searches. For the former, the criterion of minimum evolution was employed in 500 bootstrap replicates of an analysis using an empirically estimated ratio of transitions:transversions and among-site rate variation modeled as a gamma distribution with shape parameter = 0.752 using the program MEGA4 (Tamura et al.,

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TABLE I. Number of horses examined, number of horses positive, and prevalence (%) of *E. bieneusi* by location.

Location	No. of horses	No. of positives (%)
Sabana de Bogotá	60	4 (6.7)
Bogotá D.C.	25	0 (0)
Llanos Orientales	50	12 (24)
Costa Atlántica	60	5 (8.3)
Total	195	21 (10.8)

2007). For the latter, a single heuristic search under the criterion of maximum likelihood was run, using PAUP* (Swofford, 2003), employing a model selected using the Bayesian Information Content as implemented by ModelTest (Posada and Crandall, 1988). That model corresponded to HKY + Gamma, using the same gamma shape parameter, Ts/Tv = 2.7227, and relative base frequencies of 0.184 A, 0.079 C, 0.458 G, and 0.279 T.

Statistical analysis

The prevalence of *E. bieneusi* was compared between sexes and age groups. Fisher's exact test was used to analyze the data, and differences were considered significant when $P < 0.05$.

RESULTS

Of the 195 horse fecal samples examined for the presence of *E. bieneusi*, 21 were found positive (Table I). *Enterocytozoon bieneusi* was detected in 3 of the 4 geographic regions where horses were tested. The prevalence in horses <1 yr of age was significantly higher (23.7%) than the prevalence in horses >1 yr of age (2.5%; $P < 0.0001$; Table II). No differences in prevalence were observed between male (13.7%) and female horses (9%; $P = 0.3442$; Table II).

Three genotypes of *E. bieneusi* were detected by nucleotide sequence analysis of the ITS region; genotypes Horse 1, Horse 2, and D (Table III). Genotypes Horse 1 and Horse 2 are new and unique to horses, whereas genotype D has been reported in humans as well as in other hosts worldwide. When ITS nucleotide sequences identified as Horse genotype 1 were compared by BLAST analysis to other sequences in the GenBank database, it matched most closely, with 97.9% similarity, with 3 different genotypes: S5 (FJ439681) (ten Hove et al., 2009), CAF1 (DQ683746), (Breton et al., 2007), and Peru3 (AY371278) (Sulaiman, Bern et al., 2003) isolated from humans in Malawi, Gabon, and Peru. When ITS nucleotide sequences identified as Horse genotype 2 were compared by BLAST analysis to other sequences in the GenBank database, the closest matches were with 86.8% similarity to genotype S7 (FJ439683) isolated from humans in The Netherlands (ten Hove et al., 2009) and with 86.4% similarity to genotype WL6 (AY237214) isolated from muskrats in the United States (Sulaiman, Fayer et al., 2003). The Horse 1 genotype was found in 13 horses, ranging from 3 mo to 1 yr of age, in Sabana de Bogotá, Llanos Orientales, and Costa Atlántica. The Horse 2 genotype was found in 4 horses, from 3 to 7 mo of age, all in the Llanos Orientales. The *E. bieneusi* genotype D was found in 4 horses, ranging from 3 mo to 9 yr of age, in Llanos Orientales and Costa Atlántica. Mixed infections with more than 1 genotype of *E. bieneusi* were not detected.

Phylogenetic analysis was performed to determine the genetic relationship among *E. bieneusi* genotypes (Fig. 1). Although neither means of phylogenetics could fully resolve the interrelationships among such a broad array of highly similar sequences, each did identify consistent positions of the 3 genotypes isolated from horses, with respect to each other and with respect to other genotypes. Each established a close correspondence between the horse isolate identified as genotype D (GQ406055) and a broad array of minimally differentiated isolates from a broad array of hosts including, among others, humans, pigs, cats, and beavers.

TABLE III. Genotypes of *E. bieneusi* determined by sequence analysis in each positive horse are presented.

Horse ID	Location	Sex	Age	*E. bieneusi* genotype
12A	Sabana de Bogotá	Female	8 mo	Horse 1
17A	Sabana de Bogotá	Male	7 mo	Horse 1
18A	Sabana de Bogotá	Female	9 mo	Horse 1
50A	Sabana de Bogotá	Female	1 yr	Horse 1
4B	Costa Atlántica	Male	5 mo	D
6B	Costa Atlántica	Female	9 yr	D
7B	Costa Atlántica	Male	5 mo	D
37B	Costa Atlántica	Female	3 mo	Horse 1
43B	Costa Atlántica	Female	7 mo	Horse 1
12D	Llanos Orientales	Female	9 mo	Horse 1
15D	Llanos Orientales	Female	6 mo	Horse 1
17D	Llanos Orientales	Male	7 mo	Horse 2
19D	Llanos Orientales	Female	7 mo	Horse 2
21D	Llanos Orientales	Female	7 mo	Horse 1
24D	Llanos Orientales	Male	3 mo	Horse 2
25D	Llanos Orientales	Male	1 yr	Horse 1
26D	Llanos Orientales	Female	3 mo	D
28D	Llanos Orientales	Male	6 mo	Horse 2
29D	Llanos Orientales	Male	2 mo	Horse 1
30D	Llanos Orientales	Male	5 mo	Horse 1
44D	Llanos Orientales	Male	2 mo	Horse 1

TABLE II. Number of horses examined, number of horses positive, and prevalence (%) of *E. bieneusi* by age and sex at each location.

	Age				Sex			
	<1 year		>1 year		Male		Female	
Location	No. of horses	No. of positive (%)	No. of horses	No. of positive (%)	No. of horses	No. of positive (%)	No. of horses	No. of positive (%)
Sabana de Bogotá	28	3 (10.7)	32	1 (3.1)	27	1 (3.7)	33	3 (9.1)
Bogotá D.C.	1	0 (0)	24	0 (0)	1	0 (0)	24	0 (0)
Llanos Orientales	35	11 (31.4)	15	1 (6.7)	26	7 (26.9)	24	5 (20.8)
Costa Atlántica	12	4 (33.3)	48	1 (2.1)	19	2 (10.5)	41	3 (7.3)
Total	76	18 (23.7)	119	3 (2.5)	73	10 (13.7)	122	11 (9)

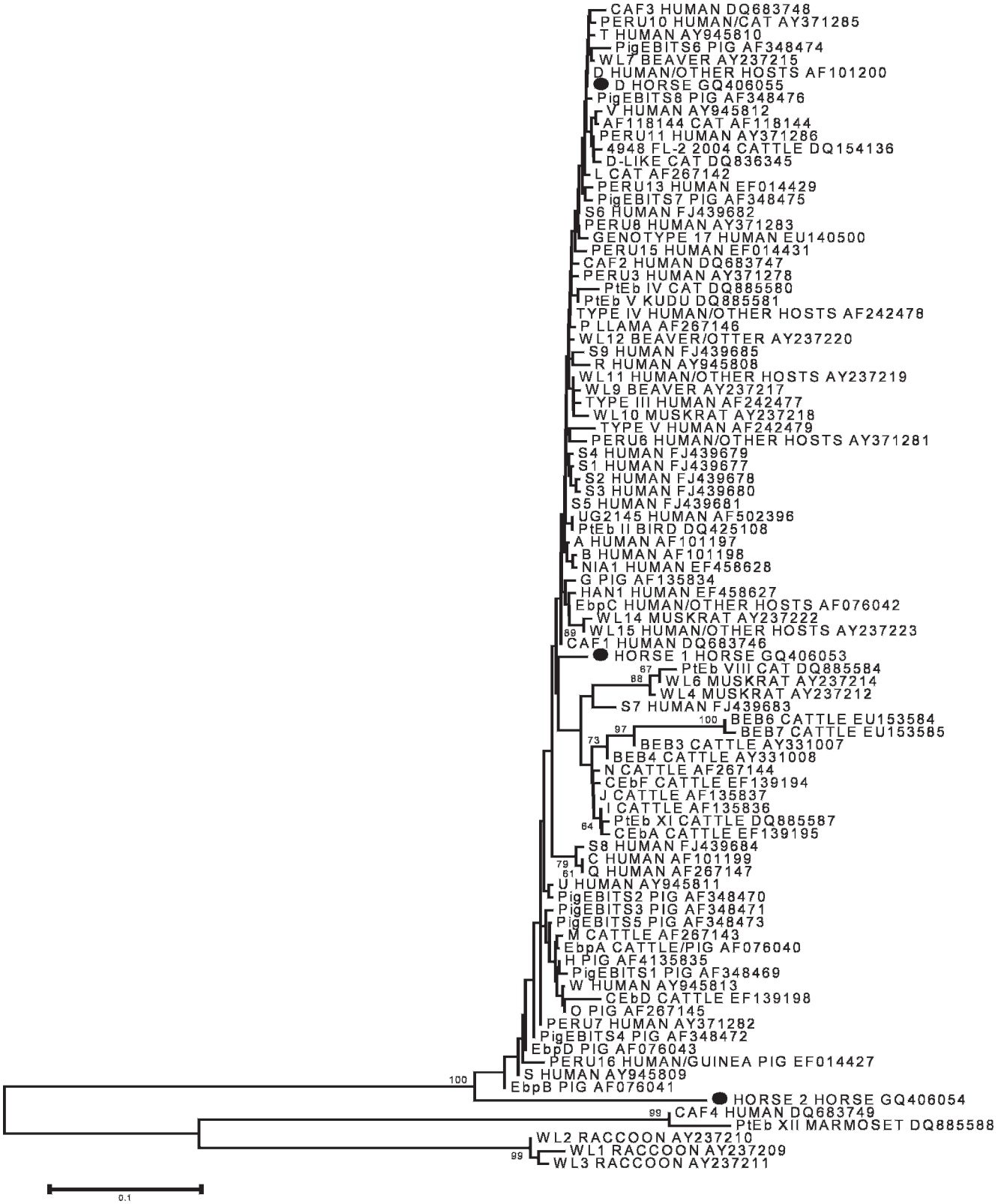

FIGURE 1. The phylogenetic position of 3 isolates from horses was investigated within a broad comparative context using the minimum evolution (ME) method (Rzhetsky and Nei, 1992). The optimal tree with the sum of branch length = 1.77301871 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.752). The ME tree was searched using the close-neighbor-interchange algorithm (Nei and Kumar, 2000) at a search level of 1. The neighbor-joining algorithm (Saitou and Nei, 1987) was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 234 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Percentage bootstrap values (>60%) from 500 replicates are shown to the left of the nodes. Nucleotide sequences determined in this study are identified with a ● before the genotype name.

Each inferred a somewhat distinct position of the Horse 1 (GQ406053) with respect to the clade described above (but with little bootstrap support for its basal position with respect to that clade). Moreover, each established a markedly distinct and basal position of the isolate from Horse 2 (GQ406054), which was excluded in all bootstrap replicates from all taxa depicted above it in the ME tree (including the members of the previously described clade and others comprised principally of isolates from cattle, human beings, and pigs) (Fig. 1). As in any such analysis of a large number of closely related taxa, the precise interrelationships among most taxa could not be resolved with any certainty (the number of rearrangements within the poorly differentiated clades is nearly infinite). However, the basic structure of variation, and the fundamental position of each horse genotype, was captured in the minimum evolution tree (Fig. 1) and in the maximum likelihood tree identified by a single heuristic search (not shown).

DISCUSSION

The present study constitutes the first report of *E. bieneusi* in equids. It was found in 21 of 195 horses examined from Colombia. Two studies examined horse fecal specimens for the presence *E. bieneusi*, but none of the 24 horses, and the 10 horses examined in Switzerland and Spain, respectively, was found to be positive (Breitenmoser et al. 1999; Lores et al., 2002). Results of the present study suggest that *E. bieneusi* could be a common parasite in horses, given that it was identified in 3 widespread geographic areas in Colombia (Sabana de Bogotá, Llanos Orientales, and Costa Atlántica). However, it probably has remained unrecognized because it is not possible to detect *E. bieneusi* by routine microscopic methods. The prevalence of E. *bieneusi* was clearly higher in horses <1 yr of age than in older horses, which could explain why no positives were observed in Bogota D.C., where only 1 horse of 25 tested was <1 yr. Although a higher prevalence of *E. bieneusi* in males than in females has been observed in dogs, cats, and fur-bearing wild mammals (Sulaiman, Fayer et al., 2003; Santin et al., 2006, 2008), no difference in prevalence between genders was observed in the present study.

Three distinct genotypes of *E. bieneusi* were detected based on genetic analysis of the ITS region of the SSUrRNA gene, 2 new genotypes named as Horse 1 and Horse 2 and genotype D (Fig. 1). Genotype Horse 1 was the most prevalent genotype identified in 13 (62%) of the 21 positive horses in the 3 regions where *E. bieneusi*-positives were detected. Genotypes Horse 2 and D were identified in 4 horses each (19%) of 21 positives. Genotype Horse 2 was identified only in Llanos Orientales, whereas genotype D was identified in Llanos Orientales and Costa Atlántica.

This is the first report of genotype D in horses. Genotype D (AF101200, DQ793213, DQ683751, DQ683755, and AF023245) was synonymized with genotypes PigITS9 (AF348477), WL8 (AY237216), Peru9 (AY371284), PtEb VI (DQ885582), and CEbC (EF139197) (Santin and Fayer, 2009) and has been reported from humans, beaver, cattle, dogs, falcons, foxes, macaques, muskrats, pigs, and raccoons (Rinder et al., 1998; Chalifoux et al., 2000; Buckholt et al., 2002; Sulaiman, Bern et al., 2003; Sulaiman, Fayer et al., 2003; Lobo et al., 2006; Breton et al., 2007; Lee, 2007; Muller et al., 2008). It is the most widespread of the genotypes of *E. bieneusi* with regard to its range of hosts and geographic distribution. It has been reported from humans in Germany, the United Kingdom, the United States, Peru, Gabon, Niger, Cameroon, Thailand, Malawi, and The Netherlands and in animals in the United States, Portugal, Abu Dhabi, and Korea (Santin and Fayer, 2009; ten Hove et al., 2009). Although the specific routes of transmission are not known, it is apparent that many wild and domesticated animals can maintain the cycle in nature. This study confirms that horses are infected with both human pathogenic and host-specific genotypes. The identification of *E. bieneusi* genotype D in horses indicates that horses could be a potential source of infection for humans.

LITERATURE CITED

Breitenmoser, A. C., A. Mathis, E. Burgi, R. Weber, and P. Deplazes. 1999. High prevalence of *Enterocytozoon bieneusi* in swine with four genotypes that differ from those identified in humans. Parasitology **118:** 447–453.

Breton, J., E. Bart-Delabesse, S. Biligui, A. Carbone, X. Seiller, M. Okome-Nkoumou, C. Nzamba, M. Kombila, I. Accoceberry, and M. Thellier. 2007. New highly divergent rRNA sequence among biodiverse genotypes of *Enterocytozoon bieneusi* strains isolated from humans in Gabon and Cameroon. Journal of Clinical Microbiology **45:** 2580–2589.

Buckholt, M. A., J. H. Lee, and S. Tzipori. 2002. Prevalence of *Enterocytozoon bieneusi* in swine: An 18-month survey at a slaughterhouse in Massachusetts. Applied and Environmental Microbiology **68:** 2595–2599.

Chalifoux, L. V., A. Carville, D. Pauley, B. Thompson, A. A. Lackner, and K. G. Mansfield. 2000. *Enterocytozoon bieneusi* as a cause of proliferative serositis in simian immunodeficiency virus-infected immunodeficient macaques (*Macaca mulatta*). Archives of Pathology and Laboratory Medicine **124:** 1480–1484.

Didier, E. S., and L. M. Weiss. 2006. Microsporidiosis: Current status. Current Opinion in Infectious Diseases **19:** 485–492.

Edgar, R. C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research **32:** 1792–1797.

Fayer, R., J. M. Trout, T. D. Craczyk, and E. J. Lewis. 2000. Prevalence of *Cryptosporidium*, *Giardia*, and *Eimeria* infections in post-weaned and adult cattle on three Maryland farms. Veterinary Parasitology **93:** 103–112.

Lee, J. H. 2007. Prevalence and molecular characteristics of *Enterocytozoon bieneusi* in cattle in Korea. Parasitology Research **101:** 391–396.

Lobo, M. L., L. Xiao, V. Cama, T. Stevens, F. Antunes, and O. Matos. 2006. Genotypes of *Enterocytozoon bieneusi* in mammals in Portugal. Journal of Eukaryotic Microbiology **53:** S61–S64.

Lores, B., C. del Aguila, and C. Arias. 2002. *Enterocytozoon bieneusi* (Microsporidia) in faecal samples from domestic animals from Galicia, Spain. Memórias do Instituto Oswaldo Cruz **97:** 941–945.

Muller, M. G., J. Kinne, R. K. Schuster, and J. Walochnik. 2008. Outbreak of microsporidiosis caused by *Enterocytozoon bieneusi* in falcons. Veterinary Parasitology **152:** 67–78.

Nei, M., and S. Kumar. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York, New York. 333 p.

Posada, D., and K. A. Crandall. 1998. ModelTest: Testing the model of DNA substitution. Bioinformatics **14:** 817–818.

Rinder, H., S. Katzwinkel-Wladarsch, A. Thomschke, and T. Loescher. 1998. Strain differentiation in Microsporidia. Tokai Journal of Experimental and Clinical Medicine **23:** 433–437.

Rzhetsky, A., and M. Nei. 1992. A simple method for estimating and testing minimum evolution trees. Molecular Biology and Evolution **9:** 945–967.

Santín, M., J. A. Cortes Vecino, and R. Fayer. 2008. *Enterocytozoon bieneusi* in dogs in Bogota, Colombia. American Journal of Tropical Medicine and Hygiene **79:** 215–217.

———, and R. Fayer. 2009. *Enterocytozoon bieneusi* genotype nomenclature based on the internal transcribed spacer sequence: A consensus. Journal of Eukaryotic Microbiology **56:** 34–38.

———, J. M. Trout, J. A. Cortes Vecino, J. P. Dubey, and R. Fayer. 2006. *Cryptosporidium*, *Giardia* and *Enterocytozoon bieneusi* in cats

from Bogota (Colombia) and genotyping of isolates. Veterinary Parasitology **141:** 334–339.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution **4:** 406–425.

Sulaiman, I. M., C. Bern, R. Gilman, V. Cama, V. Kawai, D. Vargas, E. Ticona, A, Vivar, and L. Xiao. 2003. A molecular biologic study of *Enterocytozoon bieneusi* in HIV-infected patients in Lima, Peru. Journal of Eukaryotic Microbiology **50:** 591–596.

———, R. Fayer, A. A. Lal, J. M. Trout, F. W. Schaefer III., and L. Xiao. 2003. Molecular characterization of Microsporidia indicates that wild mammals harbor host-adapted *Enterocytozoon* spp. as well as human-pathogenic *Enterocytozoon bieneusi*. Applied and Environmental Microbiology **69:** 4495–4501.

Swofford, D. L. 2003. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution **24:** 1596–1599.

———, M. Nei, and S. Kumar. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences USA **101:** 11030–11035.

ten Hove, R. J., L. Van Lieshout, M. B. J. Beadsworth, M. A. Perez, K. Spee, E. C. J. Claas, and J. J. Verweij. 2009. Characterization of genotypes of *Enterocytozoon bieneusi* in immunosuppressed and immunocompetent patient groups. Journal of Eukaryotic Microbiology **56:** 388–393.